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A ^{31}P- and ^{1}H-NMR investigation into the mechanism of bilayer permeability induced by the action of phospholipase A_2 on phosphatidylcholine vesicles

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^{31}P- and ^{1}H-NMR spectroscopy of small unilamellar phosphatidylcholine vesicles in the presence and absence of the lanthanide probe ions Pr^{3+} or Eu^{3+} have been used to study the mechanism of vesicular permeability induced by pancreatic phospholipase A_2. Monitoring the hydrolysis of PC vesicles and PC/lysoPC/oleic acid vesicles by ^{31}P-NMR indicates that all the PC molecules in the vesicles are hydrolysed and that the initial presence of lysoPC enhances the rate of hydrolysis. Studies with PC vesicles in the presence of lanthanide shift ions indicate that rapid transmembrane exchange occurs. The results are discussed in terms of the mechanism by which these transmembrane redistributions are involved in breaking the permeability barrier of the vesicular membranes, and of the application of vesicular membranes as drug packaging materials used by the oral route.

Introduction

The specific hydrolysis of the 2-acyl ester linkage of 3-*sn*-phosphoglycerides by the class of enzymes known as phospholipase A_2 has resulted in them becoming a valuable tool in probing aspects of membrane structure such as the transverse distribution of lipids across various membrane species [1]. At the molecular level, however, details are still lacking of the mechanism by which the enzyme attacks a macromolecular aggregate of lipids such as monolayer or bilayer [2]. The need for further studies of the interaction of phospholipase A_2 with phospholipid vesicular membranes is also indicated by the growing current interest in the use of these structures (liposomes) as drug microencapsulation and delivery systems [3–5].

In order to improve the design of suitable compositions and sizes of vesicular packaging for use by the oral route, methods are required to investigate the mechanisms by which the permeability barrier of the vesicles is disrupted by digestive processes. Chief among the digestive agents are the pancreatic lipases and the bile salts, and we have recently shown that the use of NMR spectroscopy in conjunction with lanthanide probe ions, can demonstrate a marked synergism in the induction of membrane permeability by phospholipase A_2 and the bile salts [6].

Previous studies have used ^{31}P-NMR to monitor the hydrolysis of phosphatidylcholine in high density lipoprotein [7] and in mixed lipid micelles [8]. However, the added use of paramagnetic lanthanide ions when studying vesicular systems allows simultaneous observation of signals from both inner and outer monolayers of the vesicles [9–11]. We are able therefore to directly follow the molecular events which precede and accompany the induction of permeability in the vesicular bi-

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Abbreviations: PC, phosphatidylcholine; lysoPC, lysophosphatidylcholine; PA, phosphatidic acid.

layers. Thus although an earlier study suggested that in the attack of phospholipase A_2 on phosphatidylcholine vesicles the products (lysophosphatidylcholine and fatty acid) were restricted to the outer monolayer [12] we conclude the these products rapidly exchange with inner monolayer lipid molecules without loss of bilayer integrity.

This insight given by the NMR technique into the molecular mechanism involved, should be a decisive value in considering which liposomal composition and structure to use as oral drug delivery systems. We report here the results obtained on the mechanism of phospholipase A_2-induced permeability of small unilamellar vesicles composed of egg phosphatidylcholine. Subsequent reports will deal with the application of ^{1}H- and ^{31}P-NMR to study the combined phospholipase A_2 and bile salt interaction and the result of using various compositions and sizes of vesicles.

Materials and Methods

Egg phosphatidylcholine was obtained from Lipid Products, (Redhill), porcine pancreatic phospholipase A_2 and 1-acyl-lysophosphatidylcholine were purchased from Sigma. AnalaR calcium chloride was obtained from BDH, praseodymium chloride (99.9%) from Lancaster Synthesis (Morecambe, Lancs), and europium chloride from Koch Light (Colnbrook, Bucks). Deuterium oxide (^2H_2O 99.8% gold label) was purchased from Aldrich.

The vesicular membranes were prepared as described previously [11] using a probe type sonicator (Soniprobe 7532A, Dawe Instruments) except that since egg PC contains unsaturated acyl chains, precautions were taken against oxidation by sonicating the egg PC and ^2H_2O under N_2 and at 4°C. The PC vesicular solution used contained 80 mg (0.104 mmoles) egg PC per ml and the vesicles composed of mixed lipid contained 0.078 mmoles PC, 0.026 mmoles lysoPC and 0.026 mmoles oleic acid (ratio 3:1:1) per ml. ^{31}P- and ^{1}H-NMR spectra of these were obtained at 37°C using a JEOL FX 90Q multinuclear FT-NMR spectrometer fitted with a calibrated temperature control and operating at 90 MHz for ^{1}H- and at 36.23 MHz for ^{31}P-NMR, respectively. The 10 mm NMR tubes contained 1 ml of vesicular solution confined by a vortex plug and capped under an atmosphere of nitrogen. The latter is to prevent oxidation from occurring. Tests of oxidation (Klein index) of lipids used in this way over several days showed that if the tubes are capped with the sample under nitrogen no oxidation of unsaturated acyl chains can be detected.

^{31}P-NMR spectra were accumulated using a total of 100 transients, employing a 15 μs, 45° pulse, an inter-pulse time of 4.5 s and a 500 Hz sweep width with 4K data points. All ^{31}P-NMR spectra were accumulated in the presence of proton decoupling. For ^{1}H-NMR spectra, 5 pulse sequences were used (π-τ-π/2) with a pulse interval of about 2 s to minimise the ^{1}H-O-^{2}H signals.

Extravesicular ionic concentrations of 3 mM Pr^{3+} (or 2 mM Eu^{3+}) and 6 mM Ca^{2+} were obtained by pipetting small volumes (approx. 10 μl) of ^2H_2O stock solutions into the NMR tubes. The required units of phospholipase A_2 were introduced into the NMR tubes by pipetting small volumes of a ^2H_2O stock solution of phospholipase A_2 (900 units or 0.726 mg protein/ml) into the vesicular solutions containing the required metal ions.

The ^{31}P-NMR spectrum of egg PC vesicles at 37°C in the presence of 6 mM Ca^{2+} includes a high resolution signal originating from the phosphate groups in the outer and inner monolayers as is shown in Fig. 1a. The corresponding ^{1}H-NMR spectrum from the same sample (Fig. 1b) includes high resolution signals from the terminal methyls (M) and methylenes (H) in the hydrocarbon chains and overlapping signals from the choline groups (C) in the outer and inner monolayer.

On adjusting the extravesicular concentration to 3 mM Pr^{3+} the spectrum in Fig. 1c is obtained by ^{1}H-NMR. This shows separate signals originating from the extravesicular headgroups O, and intravesicular headgroups I and results from a concentration-dependent downfield shift of signal O, due to the pseudo-contact shift effect of the paramagnetic Pr^{3+} in rapid exchange between the phosphate sites on the extravesicular headgroups [10]. Similar features are observed in the ^{31}P-NMR spectra where separate phosphorus signals originating from the inner and outer monolayers are obtained as is shown in Fig. 1d.

The ratio of the signal areas O/I is equivalent to the ratio of the number of choline headgroup

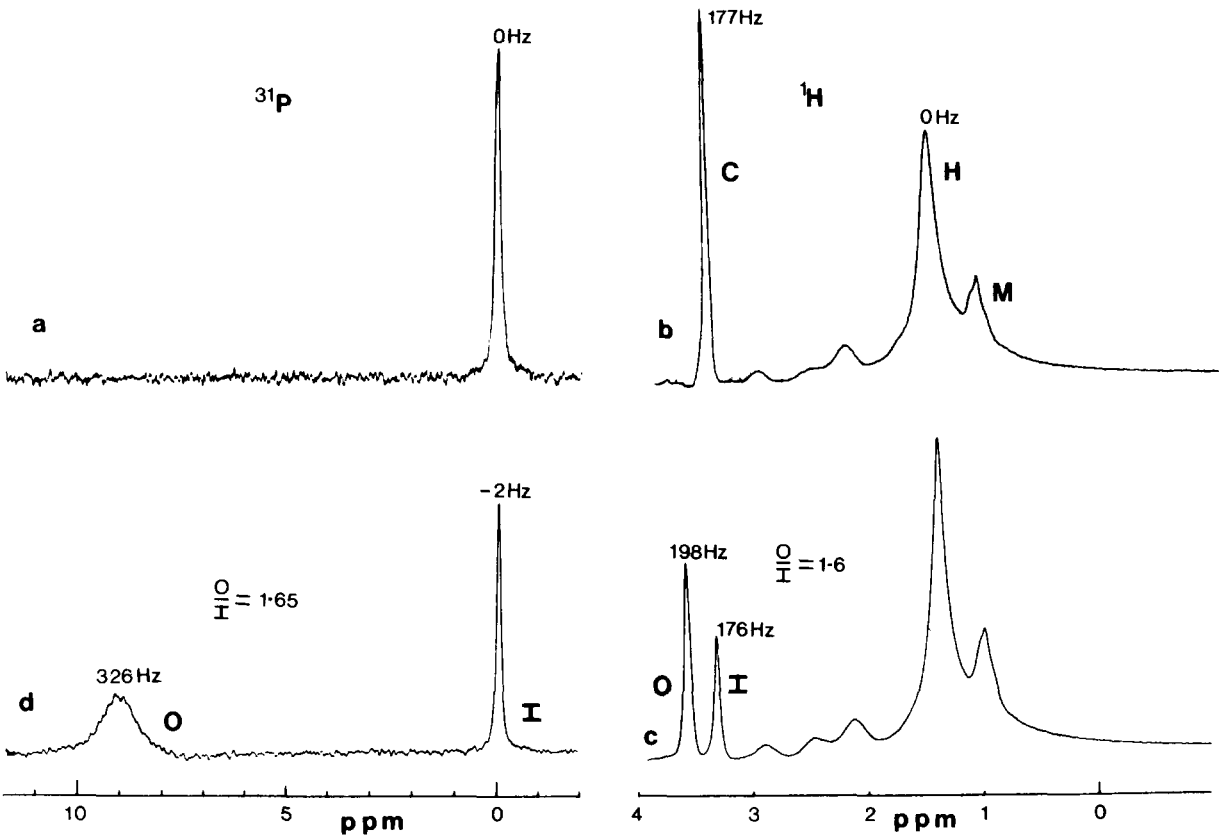

Fig. 1. NMR spectra of egg phosphatidylcholine vesicles with extravesicular Ca^{2+} (6 mM) at 37°C. (a) 36.23 MHz ^{31}P-NMR spectrum. The peak arises from overlapping signals originating from the outer and inner monolayer phosphate moieties. (b) 90 MHz ^{1}H-NMR spectrum Signals originate from the outer and inner choline headgroups (C) and methelenes (H) and terminal methyl groups (M) of the lipid acyl chains. (c) as (b) but with the addition of extravesicular Pr^{3+} (3 mM). Signals originate from the outer choline methyl groups (O) and inner choline methyl groups (I). (d) As in (a) but with the addition of extravesicular Pr^{3+} (3 mM). Signals originate from the phosphate moieties in the outer monolayer (O) and inner monolayer (I). The chemical shift scale for ^{31}P spectra in (a) and (d) are shown in ppm with respect to the single isotropic signal obtained from egg PC vesicles. The chemical shift scale for ^{1}H spectra in (b) and (c) are shown in ppm with respect to external TMS. The chemical shifts of signals O and I are measured in Hz with reference to signal H in order to compensate for paramagnetic effects on the interior of the vesicles [18].

moieties in the outer monolayer of the vesicles to that in the inner monolayer. The ratio is also a reliable indication of the vesicle size [13]. In our case the initial ratio of signal areas O/I was 1.6 indicating an average vesicle diameter of 36 nm. The O/I ratio of 1.65 obtained from the ^{31}P-NMR spectrum in Fig. 1d suggests that Nuclear Overhäuser enhancement (NOE) has little effect on the ratio of the signal areas O and I. As a precaution, spectra from a separate sample with identical conditions were obtained using gated decoupling with and without NOE, the results of which indicate that NOE has negligible effect on the signals obtained (results not shown). It has previously been shown that these peak areas can be used reliably for quantitative analysis of phospholipids present in a mixture [14].

When Pr^{3+} ions are transported across the lipid bilayer into the intravesicular solution the rise in the intravesicular concentration of Pr^{3+} causes signal I to move downfield towards signal O. By measuring the change in chemical shift (Hz) of signal I with time the rate of transport can be obtained. This method of monitoring the rates of transport is based on that described previously for ^{1}H-NMR spectra [15–17]. In order to convert

experimentally observed shifts into an intravesicular concentration of lanthanide ion (e.g. Pr^{3+}, Eu^{3+} or Dy^{3+}) a calibration graph is necessary [15]. This calibration graph (not shown) is obtained by sonicating known concentrations of the required lanthanide ion into separate vesicle preparations and then adjusting the extravesicular concentration to the required level e.g. 3 mM Pr^{3+}. At each different intravesicular concentration of lanthanide ion the shift of signal I is measured.

The chemical shifts in the ^{1}H-NMR spectra are shown in ppm with respect to external TMS. The individual shifts are shown in Hz (with respect to the hydrocarbon peak to compensate for paramagnetic effects on the interior of the vesicles [18]) for reasons of clarity in number size. In the ^{31}P-NMR of vesicular samples it is conventional to refer the ppm scale to the isotropic headgroup signal of the small vesicles [19]. All peaks are given chemical shift values with reference to this standard.

Vesicles prepared from a mixture of PC, lysoPC and fatty acid in a 3:1:1 ratio give rise to four signals in the ^{31}P-NMR spectrum (Fig. 3a), corresponding to PC and lysoPC in both the inner and outer monolayers (lysoPC 0.4 ppm downfield to PC). The production of lysoPC molecules by the interaction of phospholipase A_2 and egg PC molecules in the vesicle outer monolayer can thus be quantified from the ratio of the areas of the signals arising from lysoPC and PC in both the outer and inner monolayers.

Previous studies have shown that the relative concentrations of vesicles, lanthanide ions, calcium ions and phospholipase A_2 are critical in determining the activity of the enzyme [6]. Although Dy^{3+} was used for the ^{1}H-NMR experiments previously described [6] the use of Pr^{3+} and Eu^{3+} was resorted to in this study due to a relatively high concentration of both vesicles and phospholipase A_2 and to the line-broadening and contact-shift effects produced by Dy^{3+} in the ^{31}P-NMR spectra of the vesicles.

The experiments illustrated in this study were all performed in duplicate. They also represent a part of a large number of experiments that we have performed [6] on phopholipase A_2 and bile salt-vesicle interactions, the further results from which will be reported in later papers.

Experimental Results

Fig. 2 shows the time-dependent changes in the ^{1}H- and ^{31}P-NMR spectrum of egg PC vesicles in the presence of 6 mM Ca^{2+} and 180 units ($\equiv$ 0.145 mg protein) of pancreatic phospholipase A_2. Figs. 2a and 2b show the initial ^{31}P- and ^{1}H-NMR spectra before the addition of the enzyme (see also Figs. 1a and 1b described in Materials and Meth-

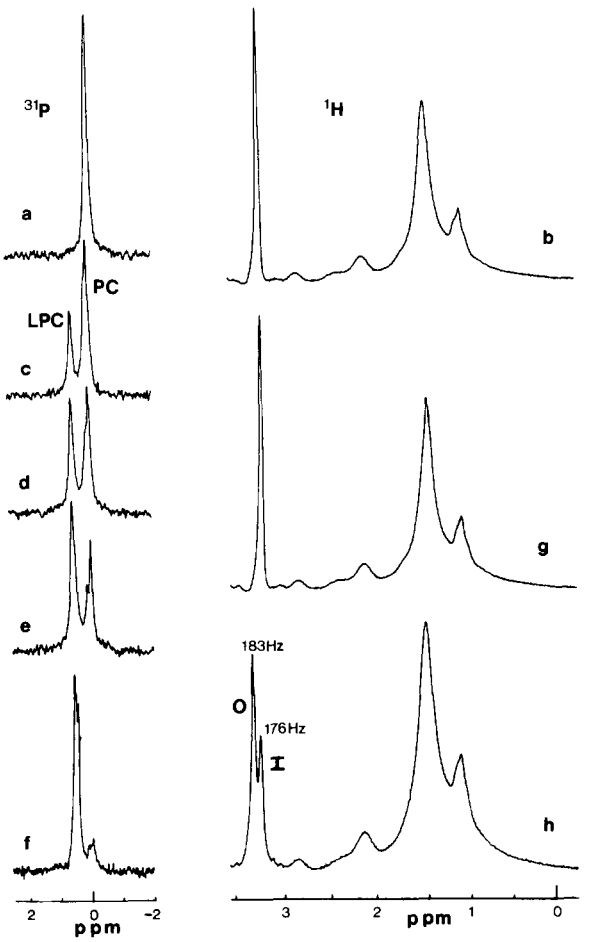

Fig. 2. (a) ^{31}P-NMR spectra of egg phosphatidylcholine vesicles at 37°C in the presence of an extravesicular concentration of 6 mM Ca^{2+}. (b) ^{1}H-NMR spectrum of the same sample. Time-dependent changes in the ^{31}P (c–f) and ^{1}H-NMR (g) signals from this vesicular solution after the addition of 180 units of phospholipase A_2. Signals are shown at the following times after the addition of phospholipase A_2: (c) 40.0 min; (d) 90.0 min; (e) 317 min; (f) 1138.0 min; (g) 1150.0 min. (h) The ^{1}H-NMR spectrum after the addition of Pr^{3+} to the sample at 1160.0 min. LPC, lysoPC.

ods). Fig. 2c shows that 40 min after the addition of enzyme a signal due to lysoPC can be observed 0.4 ppm downfield to the PC signal. Figs. 2d–f show a further increase in the intensity of the lysoPC signal at the expense of the PC signal which has almost disappeared in Fig. 2f. It is evident from these spectra that as lysoPC and free fatty acid are produced the signals arising from lysoPC and PC are both in the form of doublets.

Fig. 2g shows a ^{1}H-NMR spectrum taken after the ^{31}P-NMR spectrum in Fig. 2f. Except for some signal narrowing (probably due to the increase in bilayer fluidity) there is very little difference between this spectrum and the initial spectrum shown in Fig. 2b. It is noted that there is no significant increase in the width of the ^{1}H-NMR hydrocarbon signal during the experiment thus indicating that the hydrolysis process is not accompanied by vesicular fusion [20]. Fig. 2h shows the ^{1}H-NMR spectrum obtained after the addition of 5 mM Pr^{3+} to the hydrolysed vesicular sample. This shows separate signals from the outer and inner choline groups, indicating that vesicular integrity is retained even after hydrolysis of 95% of the PC molecules in the sample. It was also evident from the lack of formation of a ^{31}P-NMR signal at 3.5 ppm (which is characteristic of phosphatidic acid) that no phosphatidic acid has been formed during the hydrolysis experiment. This was further confirmed by TLC of the same sample which showed the presence of lysoPC, free fatty acid and some PC but no trace of any phosphatidic acid.

Fig. 3 shows the time-dependent changes in the ^{31}P-NMR spectrum of PC/lysoPC/oleic acid (3 : 1 : 1) vesicles in the presence of 6 mM Ca^{2+} and 180 units of phospholipase A_2. Here it is also seen that the presence of lysoPC and oleic acid allows the PC and lysoPC signals from the inner and outer monolayers to be resolved. Fig. 4 shows a plot of % lysoPC (derived from ^{31}P [lysoPC signal integral/total signal integral] × 100) against time. The plot gives the rate of PC hydrolysis for PC vesicles (spectra in Fig. 2) and also for PC/lysoPC/oleic acid vesicles (spectra in Fig. 3). It is evident from the plot that the initial presence of lysoPC in the bilayer increases the rate of PC hydrolysis which is consistent with the observations of Jain and co-workers [20].

Fig. 5 shows the initial time dependent changes

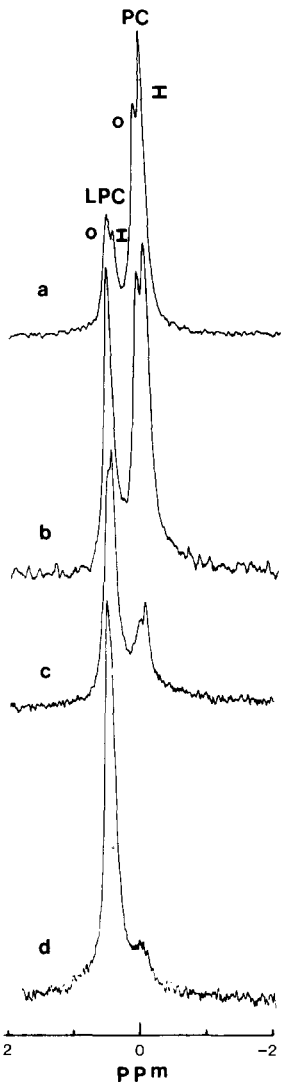

Fig. 3. (a) ^{31}P-NMR spectra of egg PC/lysoPC/oleic acid (3 : 1 : 1) vesicles at 37°C in the presence of an extravesicular concentration of 6 mM Ca^{2+}. Signals arise from the phosphorus moieties in PC and lysoPC (LPC) in the inner and outer monolayers. (b–d) Time-dependent changes in the ^{31}P-NMR signals from this vesicular solutions after the addition of 180 units of phopholipase A_2. Signals are shown at the following times after the addition of phospholipase A_2: (b) 10.0 min; (c) 85.0 min; (d) 315.0 min.

in the ^{1}H and ^{31}P-NMR spectrum of egg PC vesicles in the presence of Pr^{3+}, Ca^{2+} and 285 units (≡ 0.23 mg protein) of pancreatic phospholipase A_2. Fig. 5a shows that 3 min after the addition of the enzyme the *O/I* ratio has in-

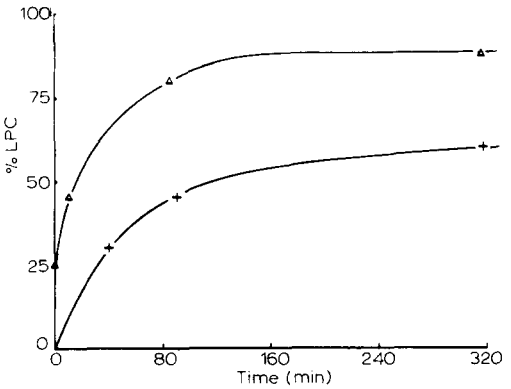

Fig. 4. Increase in the proportion of lysoPC (LPC) in the bilayer as a function of time at 37°C using 180 units of phospholipase A_2 per 1 ml of vesicle (+ ——— +, PC vesicles; △——△, PC/lysoPC/oleic acid vesicles) with an extravesicular concentration of 6 mM Ca^{2+}.

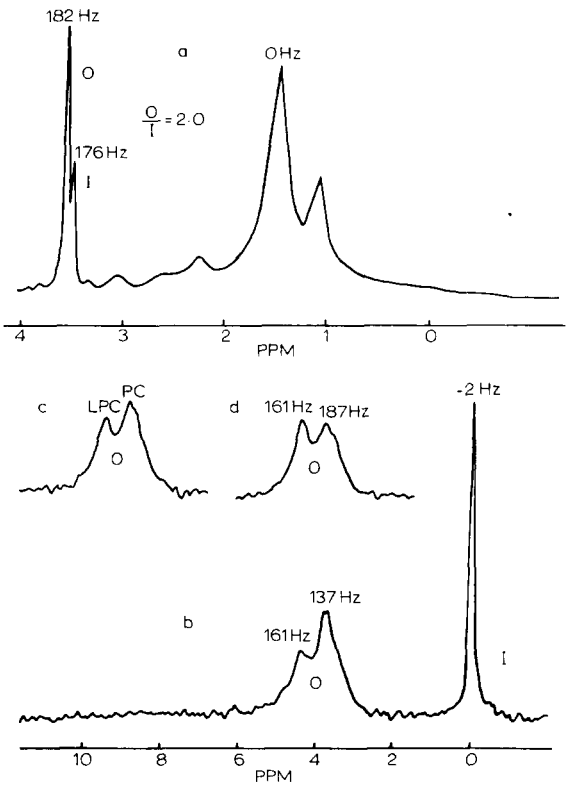

Fig. 5. Time-dependent changes in the ^1H- (a) and ^{31}P-NMR (b–d) signals from the vesicular solution illustrated in Fig. 1 (c) and (d), after the addition of 285 units per ml of pancreatic phospholipase A_2. Signals are shown at the following times after the addition of the phospholipase A_2: (a) 3.0 min; (b) 14.0 min; (c) 23.0 min; (d) 44.0 min. In spectra (c) and (d) only the signals arising from the outer monolayer are shown since no change is observed in the inner monolayer signal. See text and Fig. 1 for further details. LPC, lysoPC.

creased from 1.6 to 2.0 and that signal I has remained unshifted (compared to Fig. 1) thus indicating that no Pr^{3+} ions have permeated into the intravesicular solution. The upfield shift of signal O in Fig. 5a is due to the interaction of the enzyme with the outer monolayer headgroups and thus interfering with the interaction of the Pr^{3+} ions with these headgroups. This is substantiated by a previous study [18] which clearly shows that the shift of the outer headgroup signal O depends on: (a) shift reagent/headgroup concentration ratio, (b) the concentration and type of counter ions. Increasing the calcium concentration (added as the chloride) consistently increases the O shift due to presence of chloride ions which lowers the zeta potential and hence increases the binding of Pr^{3+} to the choline headgroup. The upfield shift of signal O seen in Fig. 5a as compared with its position in Fig. 1c (198 Hz to 182 Hz) is thus consistent with the expected effect of binding of the enzyme to the outer monolayer with a consequent increase in the shielding of the headgroup from the shift ion Pr^{3+}.

The subsequent ^{31}P-NMR spectra in Figs. 5(b–d) show the appearance of a downfield signal at 161 Hz corresponding to the formation of outer monolayer lysoPC, which is formed at the expense of outer monolayer PC molecules the signal from which (137 Hz) decreases in intensity. The upfield shift of signal O in Fig. 5b (as compared to Fig. 1d 326 Hz to 137 Hz) is as explained above for Fig. 5a. During this period only one signal, I (on the upfield side) arises from the inner monolayer phospholipid molecules. In a similar experiment but with less phospholipase A_2 it was seen that the *O/I* ratio increases from 1.6 to 2.0 as up to 25% of the outer monolayer PC molecules are hydrolysed (results not shown). Further ^1H spectra (not shown) and ^{31}P spectra (Figs. 5c–d and Fig. 6) show that no further change in the *O/I* ratio takes place as the enzyme action proceeds. However, these ^{31}P-NMR spectra indicate further increase in the intensity of the outer monolayer lysoPC and a decrease in the intensity of the outer

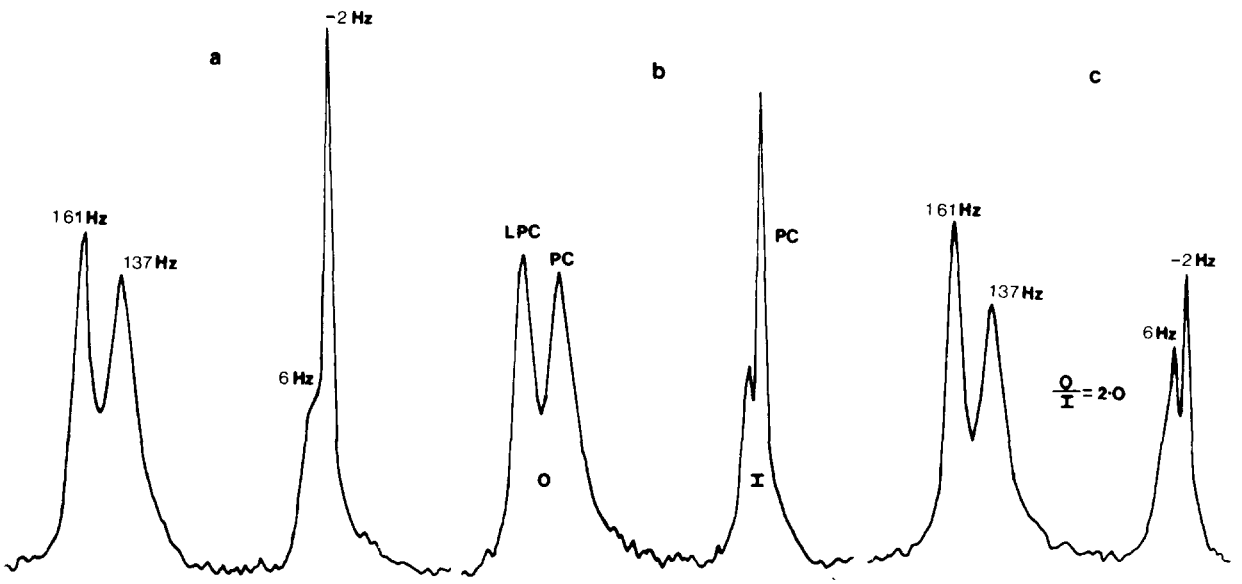

Fig. 6. Spectra (a–c) show the subsequent time-dependent changes in the ^{31}P-NMR signals to those shown in Fig. 5 (c–d). Signals are shown at (a) 101.0 min; (b) 132.0 min; (c) 220.0 min; after the addition of the phospholipase A_2 to the lipid. LPC, lysoPC.

monolayer PC signal (Figs. 5c and 5d). In addition a new peak appeares 0.22 ppm downfield from the inner monolayer PC signal (Fig. 6a). This new signal can be seen to increase in intensity at the expense of inner monolayer PC signal at -2 Hz which decreases in intensity (Figs. 6b and 6c). The spectra in Fig. 7 taken at still greater time intervals show similar features to those in Fig. 6, but in addition these spectra show the appearance of more signals downfield to the signal at 6 Hz and -2 Hz. These signals continue to shift further downfield towards signal O, but the process ceases when the signals are at about 2.0 ppm.

A similar experiment in a separate vesicle sam-

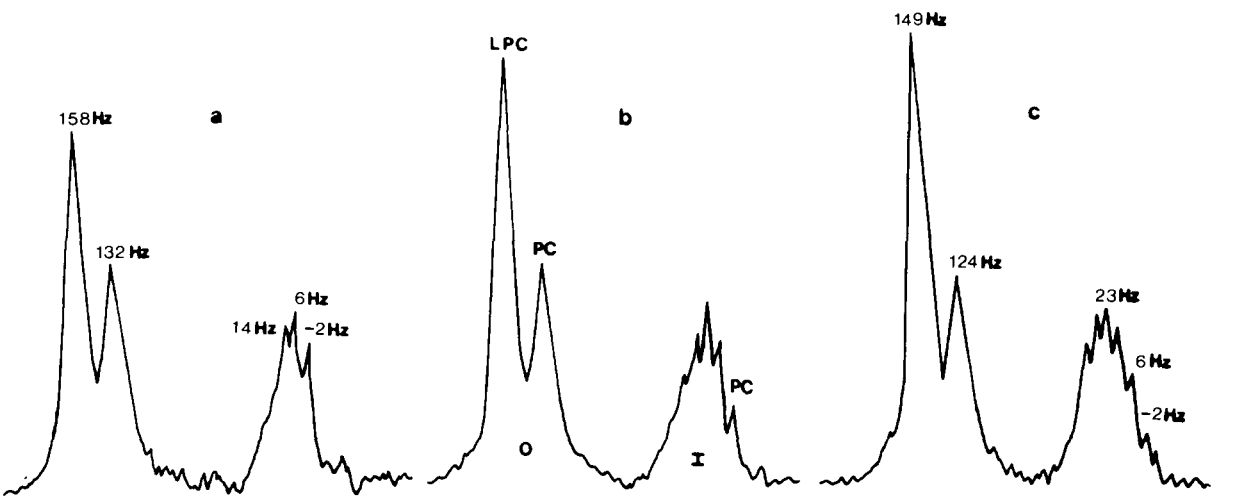

Fig. 7. Spectra (a–c) show the subsequent time-dependent changes in the ^{31}P-NMR signals to those shown in Figs. 6 (a–c). Signals are shown at (a) 305.0 min; (b) 376.0 min; (c) 850.0 min; after the addition of the phospholipase A_2. LPC, lysoPC.

ple was carried out using Eu^{3+} instead of Pr^{3+} so as to identify the nature of the peak at 6 Hz in Fig. 6. The purpose was to distinguish whether it is a signal corresponding to inner monolayer lysoPC molecules or due to inner monolayer PC molecules in a fraction of the vesicles which have become permeable to a small concentration of Pr^{3+} ($\ll 3$ mM). Fig. 8 shows the time-dependent changes in the ^{31}P-NMR spectrum of egg PC vesicles in the presence of 2 mM Eu^{3+}, 6 mM Ca^{2+} and 180 units of pancreatic phospholipase A_2. In these spectra separate signals are obtained from the outer headgroups O and inner headgroups I due to the concentration-dependent upfield shift of signal O. An initial O/I ratio of 1.6 is obtained before the addition of phospholipase A_2 (not shown). It is observed that the outer monolayer PC and lysoPC signals in Figs. 8a–d are not so well resolved as with Pr^{3+} in Figs. 5, 6 and 7. This is due to the lysoPC signal being shifted upfield by Eu^{3+} to a greater extent than the outside PC signal. Since the lysoPC signal is normally 0.4 ppm downfield from PC, this effect decreases the separation between the two signals making them less resolvable by the spectrometer.

Fig. 8b shows the appearance of a new signal at -0.38 ppm with a corresponding decrease in the intensity of the inner monolayer PC signal at 0.0 ppm. The appearence of the new peak in this region indicates that it cannot be due to lysoPC in the inner monolayer of impermeable vesicles since such a peak would occur at about 0.4 ppm. It may therefore be attributed to a fraction of the vesicles (in Fig. 8b, 20% of the vesicles) becoming permeable to a small concentration of Eu^{3+}. In both the Eu^{3+} and Pr^{3+} experiments the resulting shifted intravesicular signals are seen to occur to various degrees (Fig. 7 for Pr^{3+} and Figs. 8c–e for Eu^{3+}) indicating that permeability is being induced in different fractions of the total population of vesicles at different times. As discussed below in the Discussion it can readily be calculated from the vesicular dimensions that the small shifts observed in signal I correspond to intravesicular concentrations of only a few ions.

It is noted that during all these studies no significant change in width of the ^{1}H-NMR hydrocarbon signal was observed, indicating that the processes were not accompanied by vesicular fusion [20]. Vesicle integrity is retained even after hydrolysis of all the PC molecules in the vesicular solution since ^{1}H- and ^{31}P-NMR signals originating from molecules in the inner and outer monolayers can be obtained with no loss in signal intensity throughout each experiment. During this study a separate sample with identical conditions to the above sample containing Pr^{3+} was set up in order to investigate whether the increase in the O/I ratio was due to vesicular lysis, i.e. equilibra-

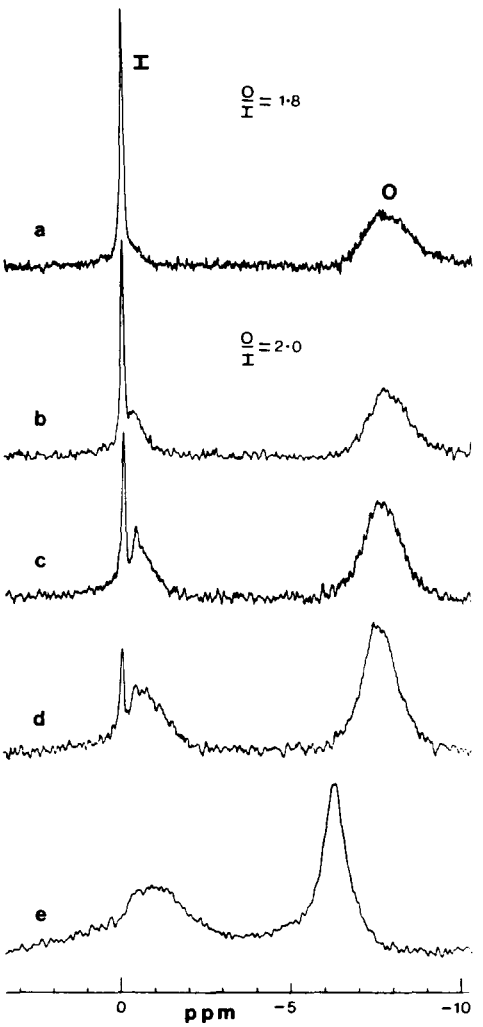

Fig. 8. Time-dependent changes in the ^{31}P-NMR signals from PC vesicles at 37°C with extravesicular Eu^{3+} (2 mM), Ca^{2+} (6 mM) and 180 units of phospholipase A_2. Signals are shown at the following times after the addition of the phospholipase A_2: (a) 18.0 min; (b) 40.0 min; (c) 65.0 min; (d) 120.0 min; (e) 4320.0 min.

tion of the 3 mM Pr^{3+} across a fraction of the vesicular population [17]. In this case a further quantity of extravesicular Pr^{3+} is added to the sample having reached an *O/I* ratio of 2.0 (as in Fig. 5a). No peak corresponding to the inside signal of lysed vesicles was revealed, indicating that no vesicular lysis has occurred.

The initial pH of each sample was 6.5 whilst the pH of samples after the hydrolysis of all the PC molecules was 4.1. This lowering of pH during hydrolysis is attributed to the formation of fatty acid. No buffers are used in these experiments in order to eliminate complications due to competitive binding of metal ions and counter ions by the vesicular system.

Discussion

The spectra in Fig. 2 indicate that all the PC molecules in the vesicular solution are hydrolysed (95% by Fig. 2f) with vesicular integrity being retained. They also indicate that the process is not accompanied by vesicular fusion. Phospholipase A_2 is too large to pass across the bilayer into the intravesicular solution (where it could hydrolyse inner monolayer PC molecules) and therefore for it to hydrolyse all the PC molecules in the vesicules transbilayer redistribution processes must be taking place; that is PC molecules in the inner monolayer are exchanging with lysoPC and fatty acid from the outer monolayer. If only the outer monolayer PC molecules were hydrolysed then a final ratio of lysoPC/PC of 1.6 would be expected. The limited shift of signal I in the sample containing lanthanide (Pr^{3+} or Eu^{3+}) indicates that the vesicles become permeable to lanthanide ions. However equilibration between the intra and extravesicular solutions does not occur since signal I stops shifting when the $[Ln^{3+}]_i$ is about 1 mM.

The mechanism by which lipid redistribution takes place in membranes has gained much attention [19]. The proposed mechanism for such lipid redistribution processes, flip-flop, involve the formation of inverted micelles. These processes normally arise from a disturbance of the equilibrium distribution of the component lipid molecules [22] and/or by the penetration of proteins giving discontinuities in the outer or inner monolayer. The composition of the molecules forming the inverted micelle determines whether the central aqueous pore is large enough to accommodate ions.

The early *O/I* ratio change seen in Fig. 5 suggests that PC molecules in the inner monolayer is exchanging with non-choline containing molecules (fatty acid) from the outer monolayer. That is two free fatty acid molecules in the outer monolayer are exchanging with each inner monolayer PC molecule [6] and that this occurs without affecting the permeability barrier properties of the bilayer to ions, since no downfield shift in signal I occurs at this stage. The process responsible for the *O/I* ratio change in this experiment occurs relatively fast (due to the high concentration of phospholipase A_2) and ceases after the hydrolysis of approx. 25% of the outer monolayer PC molecules (Fig. 5b). The priority of this fatty acid flip-flop may be a consequence of the preference of the fatty acid molecules for the more highly curved inner monolayer, or as a result of phospholipase A_2 releasing fatty acid first and only subsequently lysoPC [2]. This event probably ceases when a further increase in the proportion of fatty acid in the inner monolayer would most likely destabilise the bilayer and/or lysoPC becoming available for flip-flop. The appearence of doublets in Figs. 2d–f is most likely due to the increase in fluidity of both monolayers which would arise from the presence of lysoPC and free fatty acid. This effectively gives rise to narrower signals from both monolayers and hence the spectrometer can resolve the signals from lysoPC and PC in both monolayers (inner monolayer signals 0.15 ppm upfield to outer monolayer signals).

During this *O/I* increase in Fig. 5 the central aqueous pore of the inverted micelle may be too small to accommodate the lanthanide ions and hence the lack of transport (no downfield shift of the inner PC signal). These processes may be driven by the disruption of the equilibrium distribution of the lipid molecules in the bilayer [22] and/or by the penetration of phospholipase A_2 in the outer monolayer, since other NMR studies have shown that transmembrane migration of lysoPC in small vesicles [23] and large unilamellar vesicles [24] is catalysed by the presence of the red blood cell protein, glycophorin, in the bilayer.

Fig. 6 indicates that a proportion of the vesicles become permeable to Pr^{3+} ions and that during

this process no further change in the O/I ratio occurs. This suggests that transport may be mediated by inverted micelles composed of lysoPC and fatty acid from the outer monolayer and PC from the inner monolayer thus preserving the population of choline headgroups in each monolayer. Vesicles with lysoPC in the inner monolayer will therefore also contain lanthanide ions and will therefore have a shifted lysoPC signal. This explains why no signal at 0.4 ppm for lysoPC is observed from the samples containing lanthanide ions.

These putative inverted micelles occurring at this stage of the experiments must have large enough aqueous cavities to transport individual hydrated Pr^{3+} or Eu^{3+} ions. It can readily be calculated that vesicles of the size used here have an intravesicular volume which will contain only about 25 Pr^{3+} ions at an equilibrium concentration of 3 mM. The ^{31}P-NMR sensitivity to these concentrations is such that bursts of very few ions entering the vesicles via the inverted micelle cavity will be sufficient to produce the initial shifts in the inner monolayer signals seen in Figs. 6, 7 and 8.

These inverted micelles therefore provide a mechanism for lipid redistribution and induced permeability to proceed simultaneously. This is further substantiated by the breakdown of the permeability process as observed when signal I stops shifting. At this stage the inner monolayer is formed from only lysoPC and fatty acid and therefore no further lipid redistribution is required and the formation of inverted micelles ceases, giving rise to impermeable vesicles once again. It is of significance to note here that recent studies have shown that stable vesicular bilayer membranes can be formed when composed entirely of equimolar amounts of lysoPC and fatty acid [20,21]. We have confirmed these experiments but also observe that the vesicles composed of lysoPC and fatty acid are very permeable to lanthanide ions.

The above experiments have demonstrated that a relatively rapid rate of flip-flop occurs in small vesicles when the equilibrium distribution of lipid molecules is disrupted. Further studies employing this system with various solutes may allow the dimensions of the central aqueous pore of the inverted micelles to be determined. We will show in subsequent reports that the above methods can be applied to determine the mechanisms involved in a combined synergistic attack of phospholipase A_2 and bile salts on vesicles of different composition and sizes. The studies should be of value in producing a more systematic approach to the design of vesicular liposomes as oral drug packaging and targetting systems.

References

1 Van Den Bosch, H. (1982) in Phospholipids (Hawthorne, J.N. and Ansell, G.B., eds.), pp. 313–358, Elsevier Biomedical Press, Amsterdam
2 Slotboom, A.J., Verheij, H.M. and De Haas, G.H. (1982) in Phospholipids (Hawthorne, J.N. and Ansell, G.B., eds.), pp. 359–434, Elsevier Biomedical Press, Amsterdam
3 Ryman, B.E. and Tyrrell, D.A. (1980) in Essays in Biochemistry (Campell, P.N. and Marshall, R.D., eds.), Vol. 16, pp. 49–98, Academic Press, New York
4 Knight, C.G. (ed.) (1981) Liposomes: From Physical Structure to Therapeutic Applications, Elsevier/North-Holland Biomedical Press, Amsterdam
5 Yatvin, M.B. and Lelkes, P.I. (1982) Med. Phys. 9, 149–175
6 Hunt, G.R.A. and Jones, I.C. (1984) J. Microencapsulation 1, 113–122
7 Brasure, E.B., Henderson, T.O., Glonek, T., Pattniak, N.M. and Stanu, A.M. (1978) Biochemstry 17, 3934–3938
8 Roberts, F.M., Adamish, M., Robson, R.J. and Dennis, E.A. (1979) Biochemistry 18, 3301–3308
9 Bystrov, V.F., Shapiro, Y.E., Vikterov, A.V., Barsukov, L. and Bergelson, L.D. (1972) FEBS Lett. 25, 337–338.
10 Bergelson, L.D. (1978) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 9, pp. 275–335, Plenum Press, New York
11 Hunt, G.R.A. and Tipping, L.R.H. (1978) Biochim. Biophys. Acta 507, 242–261
12 Wilschut, J.C., Regts, J. and Scherphof, G. (1979) FEBS Lett. 98, 181–186
13 Hutton, W.C., Yeagle, P.C. and Martin, R.B. (1977) Chem. Phys. Lipids 19, 255–265
14 Dennis, E.A. and Pluckthun, A. (1984) Phosphorus-31 NMR. Principles and Applications, Chapter 14, Academic Press, New York
15 Hunt, G.R.A., Tipping, L.R.H. and Belmont, M.R. (1978) Biophys. Chem. 8, 341–355
16 Hunt, G.R.A. and Jones, I.C. (1982) Biosci. Rep. 2, 921–928
17 Hunt, G.R.A. and Jones, I.C. (1983) Biochim. Biophys. Acta 736, 1–10
18 Hunt, G.R.A. and Tipping, L.R.H. (1980) J. Inorg. Biochem. 12, 17–36
19 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 339–420
20 Allegrini, P.R., Van Scharrenburg, G., De Haas, G.H. and Seelig, J. (1983) Biochim. Biophys. Acta 731, 448–455
21 Jain, M.K. and De Haas, G.H. (1981) Biochim. Biophys. Acta 642, 203–211
22 De Kruijff, B. and Baken, P. (1978) Biochim. Biophys. Acta 507, 38–47
23 Van Zoelen, E.J.J., De Kruijff, B. and Van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 508, 97–108
24 Van Der Steen, A.T.M., De Jong, W.A.L., De Kruijff, B. and Van Deenen, L.L.M. (1981) Biochim. Biophys. Acta 647, 63–72